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STUDIES ON OXIDATION-REDUCTION

X. REDUCTION POTENTIALS IN CELL SUSPENSIONS

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STUDIES ON OXIDATION REDUCTION

X. REDUCTION POTENTIALS IN CELL SUSPENSIONS[1]

Introduction

The old device of detecting biochemical reduction by means of dye decoloration has acquired several theoretical implications. For instance, the conception of the reversible oxidation-reduction of methylene blue as a process involving the transport of hydrogen has determined the formulation of the results of several types of experiment. It has indirectly led to the use of methylene blue as a conventional substitute for ubiquitous oxygen as an oxidizing agent in anærobic experiments. Also in Thunberg's (1920) procedure for the identification of metabolites, the conduct of methylene blue is given a more or less general significance. In these and certain other uses of methylene blue are expressed, or tacit, implications.

It is, therefore, appropriate to enquire of the newer knowledge of oxidation-reduction indicators its bearing upon our understanding of cell suspensions and to examine the implications which have crept into certain current views.

In the first place the conventional "methylene blue technique" is essentially a method for kinetic studies. In order to correlate its information with that derived from the use of other indicators it is necessary to know whether any appreciable differences exist among these reagents in their resistivity to reduction from the point of view of the time required to reach equilibrium in the presence of another *active* oxidation-reduction system.

In the examination of a considerable number of dyes of the indigo, thiazine, indophenol, indamine, and meriquinone types, we have found, not only very rapid adjustments of electrode potential-differences upon partial oxidation or reduction of these systems, but also series of data which convincingly demonstrate labile equilibria among the several species of oxidant and reductant present in a given case. No better data could be required as proof that these systems adjust practically instantaneously to induced levels of oxidation-reduction intensity, which for convenience are evaluated in terms of electrode potentials.

In this paper we shall not discuss certain other reagents which exhibit distinct lag in reduction. These should not be compared

[1] Manuscript submitted for publication, Jan. 6, 1926.

with easily reversed indicators until the conduct of these less labile systems shall have been critically examined.

This mobility of the reagent systems being established, it now becomes important to determine the order in time of their reduction in cell suspensions. For, having thrown the responsibility of the well known lag in reduction upon the cell suspension, the demonstration of a correspondence between the order of the indicator systems upon the potential scale and the order of their biological reduction with respect to time will have important implications.

It has been established by experiments described in earlier papers of this series that there can be found a definite potential-difference between an electrode and a solution containing a mixture of oxidant and reductant, such as methylene blue and methylene white. This potential difference is a function of the ratio of reductant to oxidant and of the hydrion concentration of the solution. The characteristic potentials of a system indicate quantitatively its relation as oxidant or reductant to other systems which have been similarly defined.

It will therefore be convenient to speak loosely of the "potential" of a system as a numerical index of its oxidation-reduction intensity.

We need not repeat here the equations relating the variables nor the details of each of the systems examined. We shall only remind the reader that in the simple case where the hydrion concentration remains constant several of the systems may be described by Figure 1. Here the electrode potential-difference, E_h, referred to the hydrogen standard, is plotted as ordinate and the percentage reduction as abscissa. The data used in constructing this chart are for a constant pH of 7.4.

Figure 1 makes it almost self-evident that any system capable of maintaining an electrode potential of —.002 volt will maintain a 50 per cent reduction of methylene blue, will have completely reduced any of the indophenols, and will not have induced an appreciable reduction of indigo carmine. Likewise if some system shifts its reducing intensity with time and successively and in order passes through each indicator zone, it will reduce the several dyes in the order in which they are placed in Figure 1.

While the effects of various environmental factors, such as salt concentration, condensation of quinone structures with amino compounds, etc., have not been exhaustively studied, sufficient is known from the general investigations reviewed by Clark (1925) to justify the prediction that wherever a cell suspension has accomplished a definite, partial degree of reduction of one of the dyes there will be found an electrode potential-difference approximately that which can be calculated from the known constants of this dye. It could

hardly be otherwise. It, therefore, follows that potentiometric measurements will furnish greater detail and higher precision than unaided visual observations of the biochemical reduction of a dye such as methylene blue. Partial reduction which the eye fails to detect is not only made evident but mensurable. This rather restricted use of the potentiometric method in kinetic studies of dye reduction is so obvious that its proposal as a method need not be discussed in isolated detail. Examples will be found later in this paper. It is also obvious that since the potentiometric characteristics of the indicators are established, these indicators can be used alone or with potentiometric measurements in studying simultaneous equilibria between the known and unknown systems. Compare Conant & Lutz (1923), Biilmann, Jensen & Pedersen (1925), Thunberg (1925).

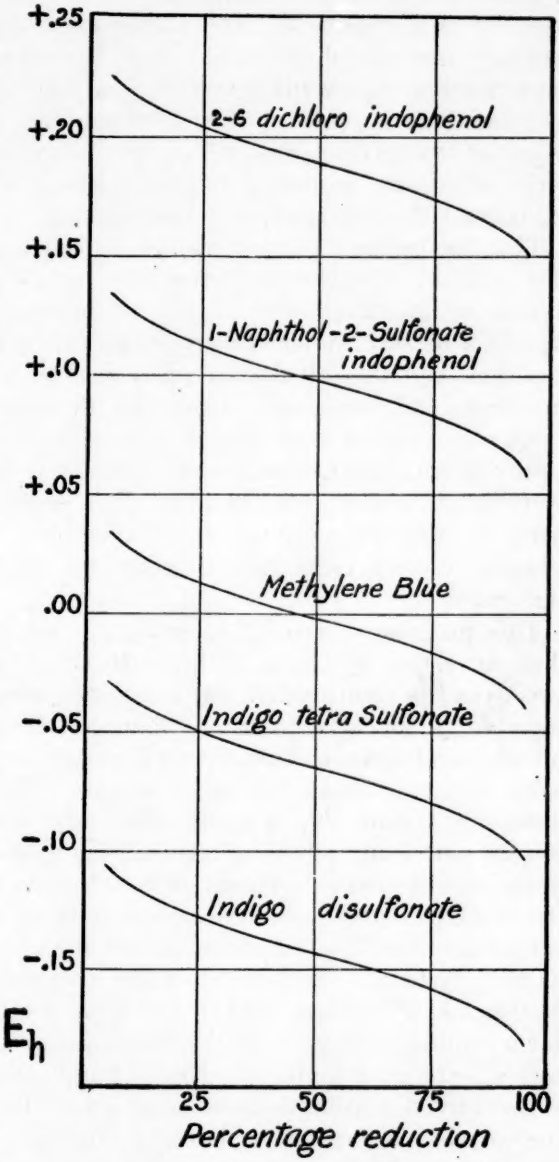

FIG. 1.—Relation of potential to percentage reduction for certain indicators at pH 7.4

The electromotive behavior of cell suspensions in the *absence* of extraneous reversible systems, such as methylene blue, would be hard to predict *a priori* from evidence at present available. There are known plenty of systems which are, in an ordinary chemical

sense, oxidation-reduction systems, but which have not yet given interpretable potentials. Such systems, exclusively, may be involved in cell chemistry, and in the absence of comprehensive knowledge it might be left to the imagination to speculate upon the mechanism whereby such systems in a cell suspension can act upon the electromotively active and reversible methylene blue system. It is for experiment to decide the very elementary but fundamental question whether there is evidence of no electromotively active systems, evidence of strongly active systems, or evidence of those intermediate types which are beginning to engage the attention of students of oxidation-reduction in organic systems.

That the systems present in cells or cell suspensions are not, in the main, of the strongly active type is a deduction that can be drawn at once from the history of biochemistry. The striking reactions of such systems would have led to their discovery, their isolation, and the solution of many problems that, under existing conditions, still await even elementary formulation.

An orderly drift toward highly negative potentials, indicative of high reduction intensities, was first observed in cultures of bacteria by Gillespie and reported in 1920. In a general way this correlates with the well-known progress of anaerobic, bacterial metabolism wherein highly reductive products are eventually liberated or elaborated.

This progressive change in potentials of bacterial cultures was then correlated by Clark (1919) with the progressive reduction of two dyes the positions of which on the potentiometric scale were reported in his 1920 paper. Potentiometric measurements in the presence and absence of the dyes were made and were found so much more suggestive than the measurements with the dyes alone that it became evident that a systematic study should be made. There were absent many of the criteria of significance upon which interpretations of electrode potentials are usually based. Consequently the biological problems were temporarily set aside during the development of indicators and of the theory of oxidation-reduction in organic systems. We hope to make it evident in this paper that neither phase of these studies has been developed as it should be for a confident attack upon the biological problems. However, certain aspects are reaching the point of applicability.

Recently, Voegtlin, Johnson, and Dyer (1924) confirmed in part the correlation mentioned above by showing a relation between the times of decoloration of several dyes in a variety of tissue suspensions and the positions of these dyes on the potentiometric scale as reported in previous papers of this series. Needham and Needham (1924) have also made some suggestive observations by means of micro-injection methods.

Of potential measurements only those of Gillespie have been published. It may therefore be well to introduce at once a very few of many measurements we have made showing the courses of potential change observed in various cell suspensions.

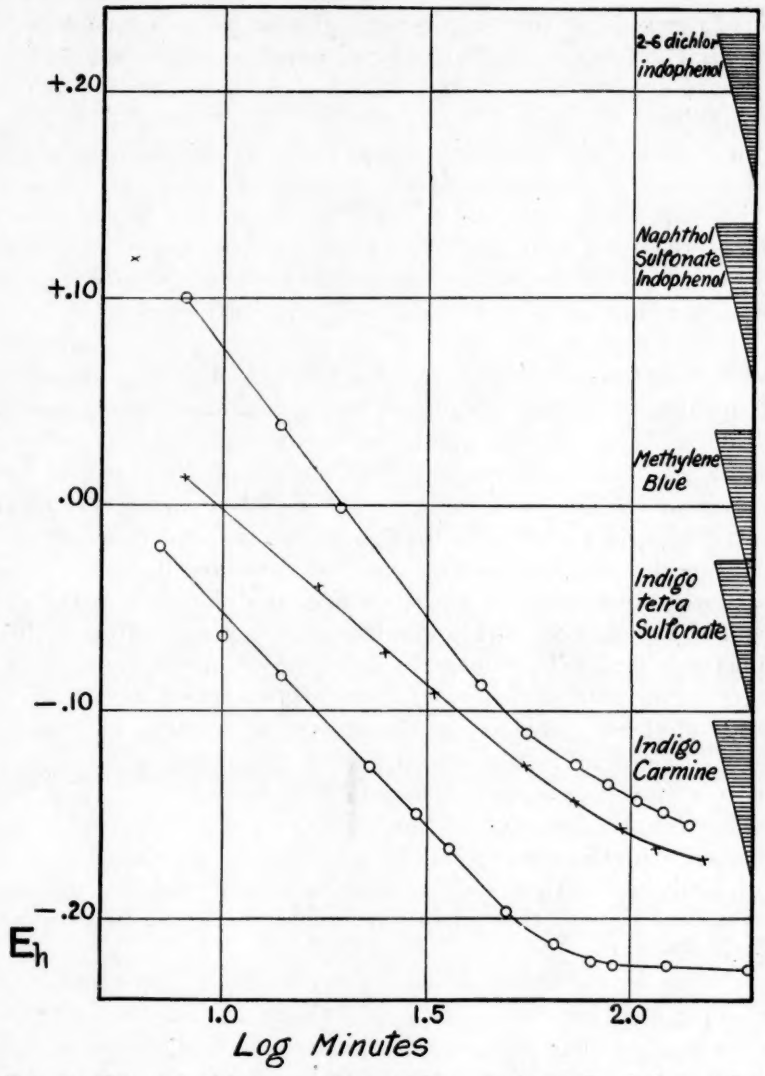

FIG. 2.—Time: potential curves of suspensions of pieces of rat liver under anaerobic conditions in a buffer of pH 7.4

In Figure 2 there are charted three series of measurements of the potentials, E_h, which were observed with suspensions of minced rat liver in a buffer of pH 7.4.

The liver of a normal rat was removed immediately after the rat had been killed. It was quickly cut into about a dozen pieces and

was then added at once to a buffer solution which had been deaerated by a vigorous stream of nitrogen. With the buffered suspension in the vessel described by Figure 1 of the third paper of this series, potential measurements were begun as soon as potentiometric balance was found, and were continued at intervals whilst the stream of nitrogen kept the suspension agitated. For convenience the potential differences at the gold electrode reduced to the hydrogen standard are the values recorded in this and in all subsequent charts and tables.

In Figure 2 the potentials are plotted against the logarithms of the numbers of minutes in order to illustrate graphically a relation which will be discussed later. At the side of this figure there are placed triangles to remind the reader of the zones of potential within which the indicators are transformed from 5 per cent to 95 per cent reduction as shown more perfectly in Figure 1.

The variation in the slopes of the curves in these experiments is attributable to various factors of which the chief is probably lack of uniformity in the subdivision or surface exposure of the liver.

Figure 3 records the results of a similar study made with a sample of dried yeast suspended in buffers of different pH values as indicated. The dried yeast was washed twice with water in the centrifuge and added as a thick, aqueous suspension, representing 0.2 gram dry material, to 50 c. c. of the deaerated buffer.

As will appear later, there are sufficient differences in the conditions of experiments with and without the presence of an indicator to make it foolish to attempt to find a direct, precise agreement between a time period required for the attainment of a given level of actual electrode potential in the absence of the dye and the time period required for the attainment of that value as indicated by the corresponding degree of actual dye reduction.

The significant aspect of these experiments is the gross correspondence in the time: potential relations of parallel experiments with and without dye such as have been observed in experiments made from time to time in this laboratory since the first report by Clark (1919).

It is to be concluded at once that electrode measurements can be substituted for indicators in the study of biological reduction. We may first note certain advantages and limitations, and then illustrate the method in rough outline by retracing ground already partly cleared by Harden and Norris (1914), Battelli and Stern (1921), Thunberg (1920), Hopkins (1921), and others.

The range of potential spanned by reasonable limits in the degree of reduction of any one indicator extends over only a portion of the range traversed by cell suspensions. Furthermore, limits to the sensitivity of the eye, and a variety of experimental factors

narrow the useful range of any one indicator to a small portion of its own span. Consequently a considerable number of indicators such as are being assembled by the work of this laboratory is required if the entire course of reduction is to be followed colorimetrically. Electrode potential measurements, on the other hand, can furnish a continuous record of the entire course.

Now the introduction of appreciable quantities of dye brings into play an *extraneous* capacity factor (poising action[1]) which is

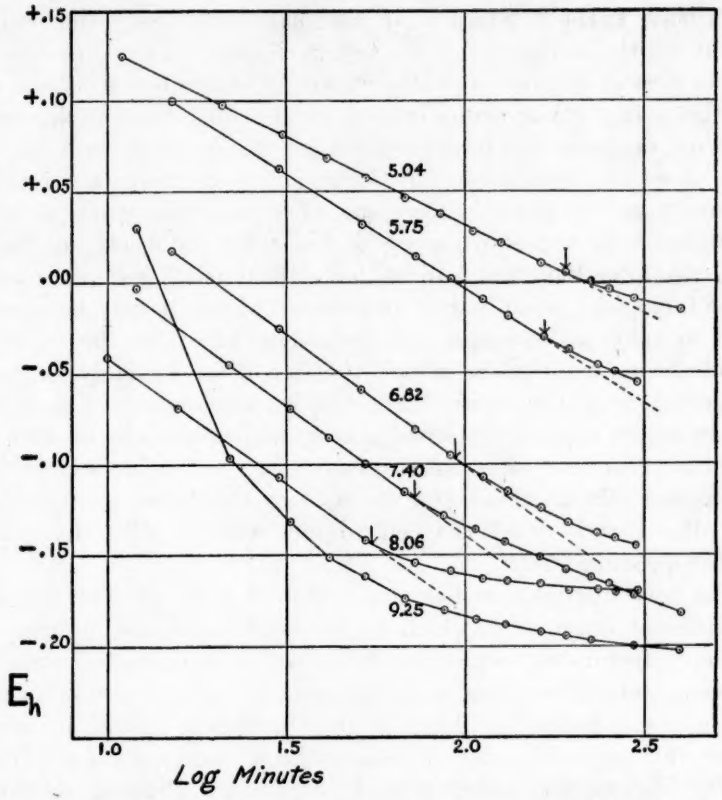

FIG. 3.—Time: potential curves of yeast cell suspensions buffered at the indicated values of pH, and under anaerobic conditions

maintained at a minimum in properly conducted potentiometric measurements.

A known and controllable capacity factor, even though it be superimposed upon an unknown quantity, contributes a certain substantiality to the indicator experiments. The work imposed becomes definite through knowledge of a definite quantity of material transformed within definite limits of reduction intensity. There have now been made available a number of useful dyes with which

[1] See Public Health Reports, 1923, **38**, p. 453.

limited potential-spans and also the quantity of material transformed may be varied. However, the experimenter should be aware of the conditions imposed in the use of indicators. Neglecting such questions as possible specific toxicity, we may call attention to the fact that the time required for reduction is not merely the time required to attain a certain level of reduction intensity with material naturally at the disposal of the cells, but it also includes the time required to attain this against the oxidizing capacity of the dye added. In the somewhat analogous case of recording acid intensities (pH) by means of acid-base indicators, the capacity factor of the added dye may ordinarily be ignored, because the dye is present in concentrations relatively insignificant. Most acids and bases in the various ionizations, hydrations, tautomeric changes and interactions which they undergo, do so with such rapidity that there is a continuous supply of active forms to accommodate instantly any slight displacement of equilibrium such as might be induced by the addition of an indicator. We are not assured that anything like this rapidity of adjustment takes place among those biological oxidants and reductants which, it may be assumed, tend to bring an indicator like methylene blue into the *temporary* simultaneous equilibrium which the dye itself is ready to follow.

Indeed, as will appear later, cell suspensions act like systems of low *active* capacity. Consequently small quantities of an oxidation-reduction indicator may become for a time relatively large in poising effect. Since this is so, does this poising effect dominate the chemistry of the cell to any extent? We shall return to this question later.

The investigator now has the choice of a number of indicators of different range with which he can study both the intensity and capacity factors of reduction; but also he can now eliminate extraneous poising systems, and by means of potentiometric measurements he can follow the detail of the changes in reduction intensity under various conditions. These conditions can be made much more nearly like natural conditions if the purely physical method be used.

Experiments on Cell Suspensions in the Presence of Indicators

Curve A of Figure 4 is constructed from data obtained with a suspension of yeast in 50 cc. buffer containing 0.00003 molar methylene blue. We may say at once that smaller amounts of the indicator are reduced without showing the plateaus we wish now to discuss. Curve B is the titration curve of methylene blue at the same pH (7.4). The reader should note the very different abscissas of these diagrams and should not be confused by the super-

ficial resemblance of the two curves. It will be observed that the correspondence between the potential zones at which plateaus appear in the two curves is reasonably good. An exact correspondence in the form of the curves would imply a constant velocity of reduction. Analysis of the curve shows that this is not the case. By use of the methylene blue titration curve the percentages of

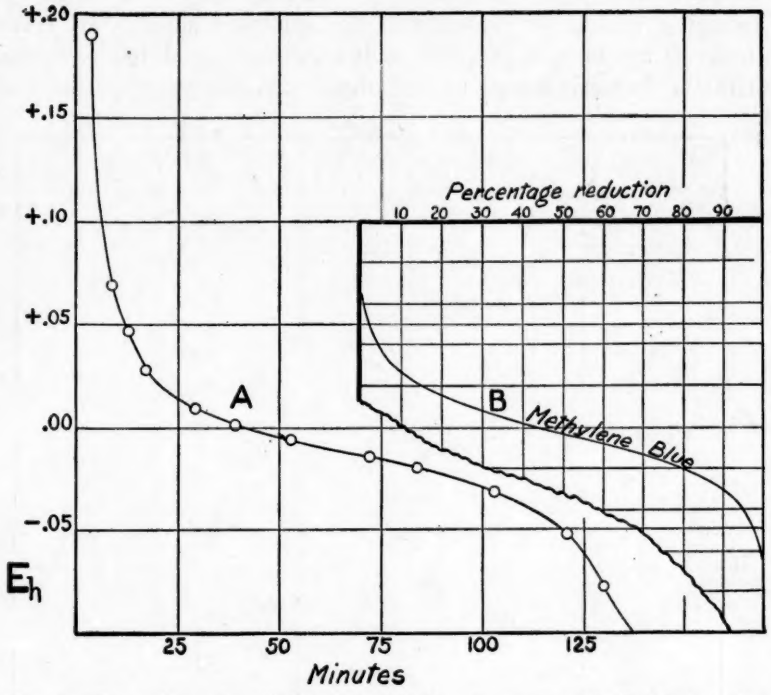

FIG. 4.—Comparison of methylene blue titration curve, B, with the time: potential curve, A, of a suspension of yeast cells containing methylene blue

reductant at various potentials are obtained. Applying these to the potentials observed in the yeast experiment we can (if the data on methylene blue in pure buffer solutions are applicable) deduce the velocity of reduction of the dye by the suspension. The results are displayed in Figure 5 and in Table 1. The "monomolecu-

TABLE 1.—*Reduction of methylene blue by suspension of yeast cells*

Time (minutes)	a	log a	a−x	log (a−x)	k×10⁴
4	97.6	1.9894	90.2	1.9552	86
16			69.9	1.8445	91
26			55.7	1.7459	94
40			42.4	1.6274	91
59			27.0	1.4314	95
71			20.1	1.3032	97
90			9.8	0.9912	111

lar" relation is betrayed not only in the curve in Figure 5 but by the velocity constants derived in the table. These experiments were not planned as exact kinetic studies and are, therefore, quoted not as examples of the degree of accuracy obtainable, but merely as illustrations of the scope of the method. In the experiments on washed tissues, to be discussed later, the same process has been applied, and it suggests a simple method for the kinetic study of the reducing ability of tissues and the catalytic activity of tissue residues. It has been the lack of such a method which has restricted quantitative investigations to the determination of the times of

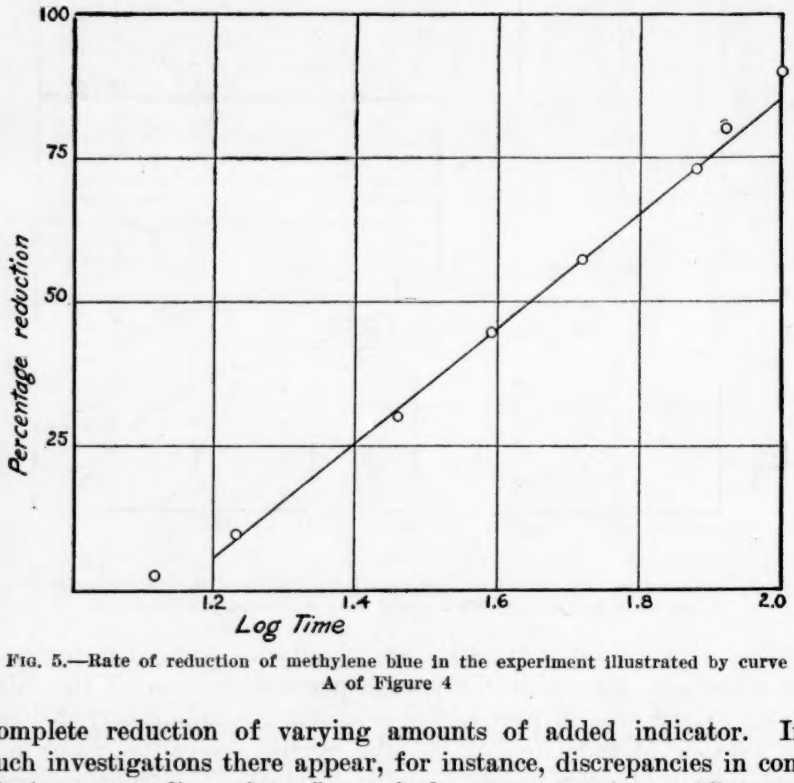

FIG. 5.—Rate of reduction of methylene blue in the experiment illustrated by curve A of Figure 4

complete reduction of varying amounts of added indicator. In such investigations there appear, for instance, discrepancies in conclusions regarding the effect of dye concentration. (Compare Voegtlin, Johnson, and Dyer (1924), and Ahlgren (1925).) Part of this is due to the failure of the method to resolve the time into that required to reach definite potentials with material naturally at the disposal of the cell suspension and that required to overcome the poising action of the added indicator.

Some cautions may be suggested. First, if much methylene blue is added, the resulting methylene white may supersaturate the solution or separate out. In this event it would be necessary to know accurately the solubility of methylene white in the medium used and

also the fact of the presence of the solid phase in order that the electrochemical equations in their numerical form might be suitably modified. Second, adsorption, for which methylene blue is noted, may appreciably influence the results. There is no evidence of this in the experiment here reported.

In earlier papers we have noted the confusion which may arise in the use of indicators the characteristics of which have not been quantitatively established. In the preceding paper of this series there were published data on systems so closely related to that of p-phenylene diamine that with reasonable approximation the gross characteristics of this system can be deduced. They are such that the system is definitely an oxidizing system with respect to the methylene blue system. Moreover, the diamines of the type of p-phenylene diamine are shown to be extremely active electromotively and certainly require no activation for their oxidation by electromotively active oxidants.

Now Szent-Györgyi (1924) recognizes, in ordinary terms, that the "hydrogen" of p-phenylene diamine is labile; and, since this implies that no activation in the Wieland sense is required, he proposes to utilize this system to demonstrate a case in which a cell suspension utilizes only its activation of oxygen. In one step of his argument he applies the Thunberg procedure and shows that washed muscle plus p-phenylene diamine does not decolorize methylene blue. In view of the fact that the p-phenylene diamine system would heavily poise the suspension in a region of oxidation-reduction distinctly positive to that of the methylene blue system, it is difficult to see any biological significance in this experiment. Had Szent-Györgyi known of the relations which are now established certain other points in his argument could have been more easily presented.

With refinement of measurements it will probably be found that certain indicators exercise more or less specific effects upon the very weakly poised cell suspensions. We have seen some suggestions of this in using methylene blue. Such effects may be sufficient to account for discrepancies in the placement of oxidation-reduction indicators on the basis of purely colorimetric and necessarily crude observations with biological material.

With this brief introductory appraisal of the indicator and potentiometric methods we may pass on to those experiments which, as we have already said, are to retrace with the aid of potentiometric measurements ground already partly cleared by others.

Experiments on Washed Tissues

It is well known from the work of Battelli and Stern (1921), Thunberg (1920), Harden and Norris (1914), and others that the

ability of a suspension of cells to reduce methylene blue is almost completely removed by exhaustive washing with water. We have followed the potentials developed when washed cells (yeast, muscle, and liver) are suspended in a deaerated buffer in the electrode vessel already referred to. The more exhaustive the washing, the less definite become the potentials, until, for example, suspensions of six times washed yeast or washed muscle hold the electrode only most erratically. The tendency toward increasing negative potentials persists; but such potentials are seriously disturbed by slight polarization of electrodes, and individual electrodes seldom come into good agreement. The levels of reducing intensity indicated by these potentials may or may not be sufficiently great to require the reduction of methylene blue, but the addition of the dye has a poising effect, and immediately carries the potential to the zone characteristic of the oxidized dye; and the subsequent reduction, as indicated by the potential-change is very slow.

When there is added to such a suspension of washed tissue a deaerated solution of one of Thunberg's "metabolites" (e. g., sodium succinate) in the same buffer, the electrode registers a rapid trend toward increasing negative potentials and at the same time the potentials are less disturbed by mild polarization and recover rapidly thereafter. Individual electrodes also come into much better agreement. The main lines of such an experiment (in this case with yeast) are depicted in Figure 6. The effect of the addition of the oxidizable substance upon the ability to reduce methylene blue was also indicated by the potential-change when that dye was added. If there be derived from the latter the curve of the velocity of reaction it will be found to have the exponential form. Sometimes the velocities calculated with the monomolecular formula come out remarkably constant, but sometimes, as Table 2 shows, there is a small but distinct tendency for the values of k to fall as reduction proceeds.

TABLE 2.—*Reduction of methylene blue by washed yeast cells in the presence of succinate*

Time (minutes)	a	log a	a−x	log (a−x)	k×10⁴
3	92.5	1.9661	86.9	1.9390	90
5			82.0	1.9138	105
7			77.8	1.8910	107
9			74.5	1.8722	104
13			67.6	1.8299	105
18			61.7	1.7903	98
23			55.7	1.7459	96
33			47.5	1.6767	88
44			39.8	1.5999	83
52			35.2	1.5465	81
64			29.5	1.4698	78
82			21.6	1.3345	77
99			12.6	1.1004	87

Further work may show that the levels of reducing intensity attained in suspensions of washed tissue plus different substances are characteristic of the substances under specified conditions. So far as we know, such characteristics could hardly be compared with the characteristic equilibrium constants applicable to definite reversible systems. Nevertheless, as empirical data of use in the study of intermediary metabolism they should be more valuable than the empirical classification of metabolites according to whether they do or do not reduce methylene blue in the presence of washed tissues. Were indigo carmine used in place of methylene blue a much more restricted list of "metabolites" would be found; whereas, if an indophenol were employed, their name would be legion. Washed tissues which fail to reduce methylene blue still actively reduce 2,6-dichlorphenol indophenol, do so in the presence of molecular oxygen and retain this ability after boiling. In the latter case the reducing

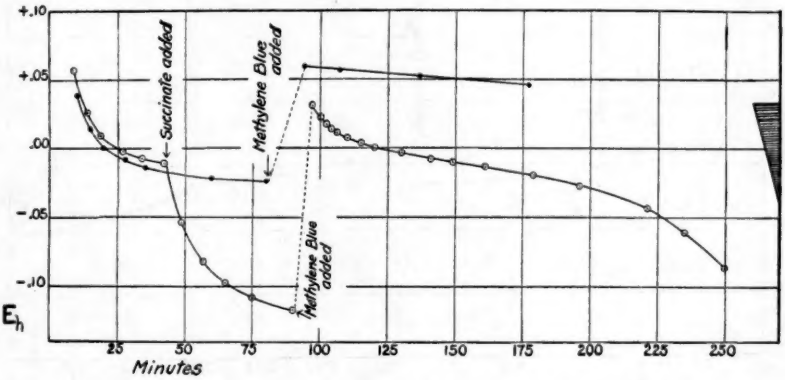

FIG. 6.—Effect of a "metabolite" on the reduction of methylene blue by washed yeast cells

capacity is limited, can be largely removed by alcohol extraction, and may, in part, represent the thermostable "hydrogen donators" of Hopkins. This possibility is supported by the fact that the ability of boiled washed yeast to reduce this indicator is not impaired appreciably by two hours' aeration at pH 7.4, but is notably reduced in amount if glutathione is added before aeration.

The Glutathione System

It has already been stated that boiled, washed tissue reduces indophenol, and it was suggested that this may be related to the thermostable "hydrogen donators" of Hopkins. There may now be described a few experiments which demonstrate potentiometrically certain features of the relations of glutathione to tissue "respiration."

One-half gram of washed yeast was boiled in water for two

minutes, washed twice more with water, and then added to 50 c. c. of buffer in the electrode vessel. The potentials observed were as unsatisfactory as those found with washed tissue. When methylene blue was added, the potential jumped to that of the oxidized dye system, and the change with time indicated only a very slow reduction (within 6 per cent) of the dye. These results are shown in curve A of Figure 7. The further addition of a deaerated solution containing 5 mgm. of oxidized glutathione resulted in a much accelerated reduction of the dye. In Table 3 we have calculated from the potentials observed in the presence of the dye the rates of reduc-

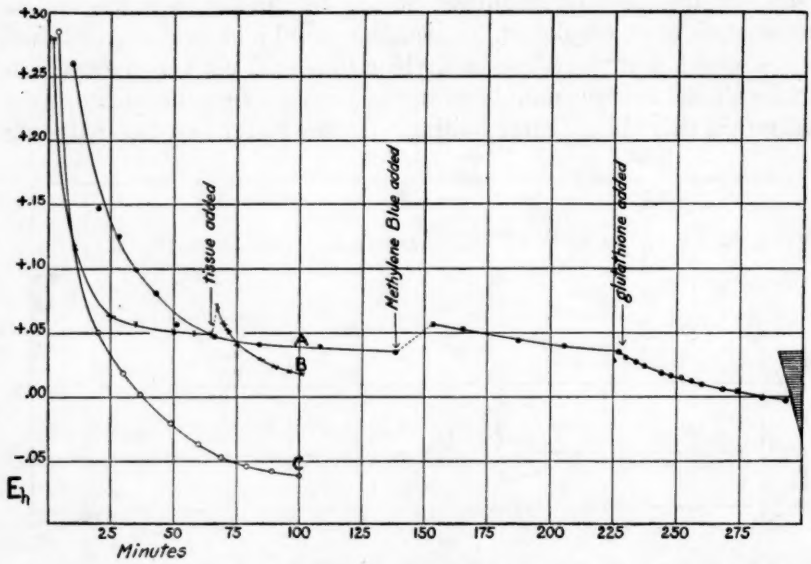

FIG. 7.—Curve A—Effect of glutathione on the time: potential curve of washed, boiled cells in the presence of methylene blue. Curve B—Time: potential curve of glutathione-methylene blue mixture before and after addition of boiled, washed yeast cells. Curve C—Time: potential curve of suspension of yeast cells containing initially, oxidized glutathione

tion by the yeast alone and by the yeast in the presence of the glutathione. The acceleration is made evident and the reaction rate in this case appears to be constant rather than monomolecular. Curve B is the control experiment, in which the rate of reduction of the methylene blue by the glutathione alone is compared with that on the subsequent addition of boiled, washed yeast. The rates of decolorization observed can scarcely be compared with those found by Hopkins as the preparation of glutathione at our disposal was of uncertain quality and the concentration of tissue in the suspension was much less than that used in the latter worker's experiments.

TABLE 3.—*Reduction of methylene blue by boiled, washed yeast cells in the presence and absence of glutathione*

(CURVE A.) IN THE ABSENCE OF GLUTATHIONE

Time (minutes)	a	a−x	log (a−x)	k×10[4]
22	98.4	96.8	1.9859	32
40		95.5	1.9800	32
52		94.15	1.9738	37

(CURVE A.) IN THE PRESENCE OF GLUTATHIONE

Time (minutes)	Amount changed	Amount changed per minute	Time (minutes)	Amount changed	Amount changed per minute
	Per cent	*Per cent*		*Per cent*	*Per cent*
4	2.15	0.54	30	20.5	0.68
7	4.6	0.66	38	25.7	0.67
14	9.4	0.67	44	30.6	0.69
18	11.4	0.63	54	38.3	0.70
22	14.3	0.65	63	40.5	0.63
26	17.2	0.66			

(CURVE B.) CONTROL EXPERIMENT. REDUCTION OF METHYLENE BLUE AFTER ADDITION OF WASHED, BOILED CELLS

3	2.2	0.73	25	15.0	0.60
13	7.8	0.68	29	17.3	0.60
18	11.2	0.62			

Curve C of the same figure provides the complementary data of the effect on the potentials of the addition of oxidized glutathione alone to the suspension of cells. The potentials then obtained become much more certain than with the suspension alone, and electrodes come into good agreement. The limiting potentials so achieved probably agree with those characteristic of a solution containing reduced glutathione and are of the same order as those reported by Dixon and Quastel (1923). The latter authors' data are difficult to formulate electrochemically, but evidently indicate the potentials of reduced glutathione systems, so that our curve C seems to indicate definitely the reduction of glutathione by the suspension. In other words, the electrode agrees in the conclusion that cells contain thermostable "hydrogen donators" which are able to reduce glutathione and, consequently, to reduce methylene blue. Much more might be done with the electrode in confirming the main features of the glutathione respiratory system. Suffice it now to add the reflection that the potentials of the methylene blue system peculiarly adapt it as an indicator for this biological process. A dye somewhat more negative in characteristic potential would not be reduced by glutathione plus boiled, washed cells, while such a dye as indophenol is reduced so rapidly by the washed cells alone that it would be difficult to demonstrate an acceleration in the presence of glutathione, if such occurred.

Potentials in Growing Cultures of Bacteria

The form of the time: potential curve for a growing culture of bacteria is determined, not alone by the unit activity of the specific cells under observation, but also by enormous changes in the number of active cells. Consequently the curves of Figures 8 and 9 are not directly comparable with those of dense suspensions of preformed cells.

Figure 8 illustrates a fact first noted by Gillespie (1920), namely, that cultures of different species run different courses and attain dif-

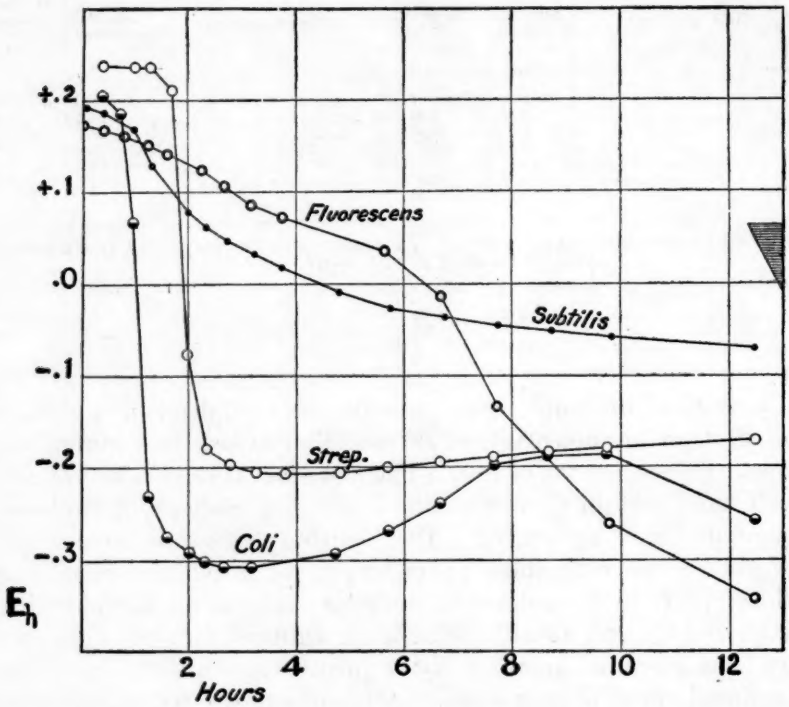

FIG. 8.—Comparison of time: potential curves of *Bact. coli, Strep. lacticus, B. subtilis,* and *B. fluorescens* in milk at 37° C

ferent levels. In the cases illustrated here the cultures were in milk at 37°C., and the different species induced very different changes of pH. Consequently, certain details of the curves are not directly comparable. Nevertheless, they may serve for the present as illustrations of a considerable number of experiments with a variety of species and several different media—all tending to the conclusion that systematic surveys will reveal theoretically important features in these time: potential curves. Certain practical applications are obvious. Of interest for the moment is the fact that the range

of potential revealed by the potentiometric method is very much greater than could be suspected from the conduct of one indicator. A reminder of the zone of potential within which methylene blue is transformed from 5 to 95 per cent reduction at the pH of fresh milk is the triangle in Figure 8.

It is true that the potentials of bacterial cultures, especially in the early stages of growth, are not well poised. Consequently, different electrodes in the same culture frequently show decided differences; but, as the characteristics of definite reversible systems, notably the indicators, become defined and their conduct in cultures is compared with potentiometric measurements, a real significance in the latter becomes more evident.

Figure 9 is rather interesting for two reasons. In the first place curve A is a transcript to new coordinates of the first section of a

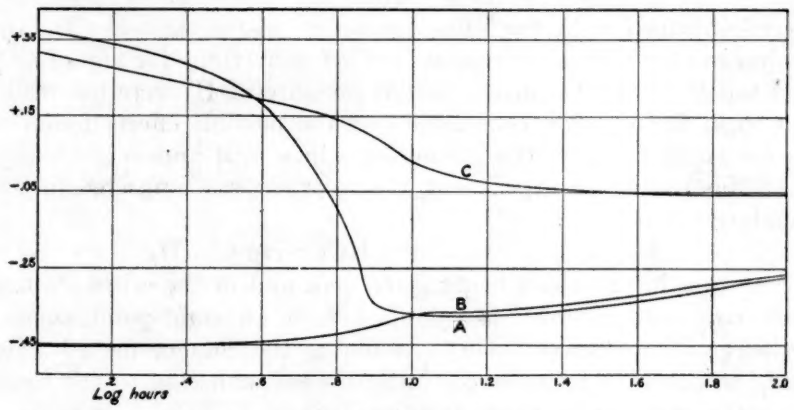

FIG. 9.—Transcript of time: potential curves traced by a Leeds and Northrup recording potentiometer. A—*Bact. coli* strain *fg*; C—*Proteus vulgaris* strain *acx*; B—Hydrogen electrode potential of the *coli* culture

record showing the potential changes in a culture of *Bact. coli* as traced faithfully over 5.5 meters of record for three days by a Leeds and Northrup recording potentiometer. In the second place the records of "reduction potential" and hydrogen electrode potential show what we regard as a fact of considerable importance.

The organism was grown in a medium consisting of 0.5 per cent each of Witte peptone, K_2HPO_4 and d-glucose. The incubation temperature was 30° C. Four liters of the medium were held in a five-liter flask. Initially, then, there was approximately a liter of moist air above the culture. Variations of gas pressure were accommodated through a tube filled with sterile cotton. When the culture began to "gas," some of the unabsorbed oxygen must have been displaced, but at no time could a gas pressure exceeding the atmospheric have been developed. One siphon, plugged at its end

with sterile agar saturated with KCl and containing crystals of KCl, dipped into a solution of KCl in the presence of the solid phase and through this solution was made the liquid junction with a "saturated" calomel half-cell. Another siphon provided the means of withdrawing samples of the culture from the neighborhood of the platinized platinum electrode. These samples were used in the determination of the potential of a platinized electrode under one atmosphere partial pressure of hydrogen—the hydrogen electrode potential. Such determinations were made directly in all important cases, and through colorimetric measurements of pH values at frequent intervals. The course of these potentials is traced by curve B. In the reduction of observations to E_h values shown in Figure 9, proper corrections have been made for the difference of potential between the calomel electrode of the oxidation-reduction chain and that of the hydrogen electrode chain. All important measurements were confirmed with the same precision potentiometer. In the hydrogen electrode measurements *refined* corrections for loss of CO_2 and the effect of CO_2 on the partial pressure of H_2 were not made, but these factors were considered and the possible effects found to be too small to affect the conclusion which will appear presently.

In this particular experiment, the recorder was connected to two separate chains:

Pt, culture : saturated KCl : Hg_2Cl_2, Hg

the culture in one chain being *Bact. coli*, and in the other *Proteus vulgaris*. The recorder is equipped with an eight-point contact switch; and to prevent short circuits as the contact moved from point to point, odd numbered contacts were connected to one chain and even numbered contacts to the other.

The chain containing the culture of *Proteus vulgaris* induced the comparatively slightly negative E_h values shown by curve C of Figure 9. Consequently, when the contact of the recorder moved from points on the switch which were connected to the "*proteus*-chain" to those which were connected to the "*coli*-chain," the potentiometer was considerably out of balance with the latter. As a consequence the electrode in the culture of *coli* received once every 50 seconds a small but nevertheless distinct "depolarizing" current during the automatic adjustment of potentiometric balance. This tended to make the E_h values more positive. The importance of this experimental condition will presently be apparent.

While no analysis of the gas over the culture was made in this particular experiment, it is known from the study by Rogers, Clark, and Davis (1914) that this *fg* strain of *Bact. coli* produces under strictly anaerobic conditions in the medium here employed a somewhat larger volume of carbon dioxide than of hydrogen. It is also known that under conditions not strictly anaerobic the proportion

of hydrogen is lower. It is thus evident from the experimental conditions described above that a partial pressure of hydrogen of a full atmosphere could not possibly have been produced.

Finally, then, it is evident from the crossing of curves A and B of Figure 9 that, in spite of slight but repeated "depolarizing" currents received by the electrode, there developed a definite "overvoltage" which, once attained, was maintained during the acidity change. This is not an isolated instance, for we have observed it many times. It is, moreover, an experiment made under convincing circumstances.

We have a case in which the addition of asparagine, which is known to suppress the evolution of hydrogen, resulted in a potential slightly positive to that of the hydrogen electrode. We also have cases in which organisms of the dysentery group in media from which they produce no hydrogen, brought the potential almost to that of the hydrogen electrode. But these observations can be adequately appraised only after more systematic surveys.

We believe that there are important facts to be found in the relation of these time: potential curves to the growth curves of all living cells. Because of the arduous nature of the growth curve experiments and the fact that we are not yet equipped to handle them efficiently it will be some time before we shall feel confident in reporting upon work already done and work contemplated. Therefore, we feel justified in making what we believe *should be a very unusual request*, namely, that this phase of the subject be reserved to us.

Oxidation-Reduction Intensities of Cell Suspensions in the Presence of Oxygen

Although the experiments reported up to this point were not designed to show clearly the potentials of dense cell suspensions immediately after or during exposure to air, the thoughtful reader may have been surprised to note that the initial potentials recorded are extremely negative to the potentials of the hypothetical "oxygen electrode."

It is well known that the oxygen electrode behaves as if it were not truly reversible and, until its conduct is thoroughly understood, experiments with electrodes in the presence of atmospheric oxygen must be considered with caution. However, the electrodes in these cases behaved somewhat as they do in a variety of solutions which are but slowly oxidized by air, but which, in the presence of air, are able to induce definite potentials negative to that of the hypothetical oxygen electrode. Indeed, there is no need to exclude air in crude measurements of some reversible systems—a fact which indicates the comparatively inert nature of molecular oxygen.

The presumption, which could not be accepted without definite confirmation, is that the electrode by its indications in aerated cell suspensions is tending to record the activity of some system lying between +0.1 and +0.2 volts at pH 7.

This suggestion is rendered somewhat dubious by the fact that potentials observed under these circumstances hover not far from the region of the unpoised potentials of sterile culture media and similar solutions. We shall not now pause to discuss this interesting phenomenon, for the potentials of the aerated cell suspensions seem to be rather more definite and are in essential agreement with a fact upon which we commented in the sixth paper of this series: "*The fact that all living cells tested reduce this dye* [2,6-dibromindophenol] *indicates not that we have reached one limit of endurance, but that we are perhaps approaching one limit of normal physiological oxidation-reduction intensity; and it is of very considerable importance that this limit lies midway in the possible range.*" We now find that a suspension of washed cells which is unable appreciably to reduce methylene blue under anaerobic conditions will reduce and maintain reduced 2,6-dichloro indophenol, even though a vigorous stream of air be passed through the suspension. While being aerated, such a suspension will have but comparatively slight effect on 1-naphthol-2-sulfonate indophenol and, of course, will not reduce methylene blue.

Now the reduction of a dye involves work. The fact of reduction in the case of the first indophenol is therefore evidence of a very definite activity on the part of the cell suspension. It would be beside the point of the present discussion to consider localities of reduction or to take up problems which arise when the heterogeneity of the suspension is considered. It need only be assumed that any possible partition coefficients of the several forms of the dyes are such that the order of magnitude of the oxidation-reduction intensity is indicated by the presence of appreciable amounts of the reductant of the dichloroindophenol and of the oxidant of the indonaphthol. On this assumption, which seems fully justified by the color of the aqueous phase, the indicator and electrometric measurements are in rough but substantial accord.

We are not competent to judge certain aspects of the micro-injection experiments of Needham and Needham (1925); but their conclusion and ours seem to be in essential agreement. They define the oxidation-reduction intensity of living *Ameba proteus* to lie between rH 17 and 19; but they emphasize the possibility that a conclusion drawn from the study of one organism may not be of general significance. Our discussion deals, not with an attempt to define so definitely, but only with the proximate position of the zone of

oxidation-reduction intensity in aerated suspensions of several different cells.

One aspect of Needham and Needham's work is in agreement with a very considerable number of loosely formulated observations recorded in the literature, namely, that cytolysis and injury are accompanied by what the Needhams call a "burst" of reduction. Since the cells in our experiments obviously were under abnormal conditions, it is necessary to consider the possibility that our indicator and potentiometric studies were revealing initial stages of this "burst" of reduction and that the intact, aerated cell is decidedly more positive than the data seem to indicate. We were, therefore, anxious to apply the meriquinones, the formation of which seemed at the beginning of our investigation to be induced by oxidizing systems distinctly positive to the zone characterizing the indophenols; and we delayed the publication of this paper until the investigation of the meriquinones, reported in the preceding paper of this series, was sufficiently complete for the purposes of the present discussion.

Unfortunately the systems resulting from partial oxidation of p-phenylene diamine, benzidine, and their homologues are so very unstable and the equilibria involved are so complex that we were forced to the conclusion that these reagents are unreliable, and their use is prohibited in *precise* investigations of biological material. Recognizing this we may, nevertheless, draw some conclusions regarding the gross aspect of their conduct in cell suspensions.

In the paper referred to it was shown that the high association constant of the meriquinone of p-aminodimethyl aniline (and presumably of p-phenylene diamine), when combined with the effects of the pH value of the solution, concentration of total oxidant and reductant, and other factors, will permit appreciable oxidation of these systems at an induced potential characterizing the indophenols. If the potentiometric and indicator results for aerated cell suspensions stated above are correct, then the aerated cell suspensions should induce the oxidation of p-phenylene diamine. Battelli and Stern (1912) state: "Wir werden sehen, dass alle Tiergewebe die Fähigkeit aufweisen, das p-Phenylendiamin zu oxydieren." Since we are concerned only with the gross aspects of the subject we shall not discuss limitations to this statement either from the point of view of limitations in the abilities of cells or from the point of view of the design of reagent solutions.

In contrast to systems of the phenylene diamine series are those of the benzidine series. Here the lower limits of solubility limit the design of the reagent solutions, and this combined with the lower meriquinone association constants and various other factors conspire to restrict the range of oxidizing intensities which can induce appreciable formation of a true meriquinone color. A sys-

tem possessing the oxidizing intensity suggested by the electrode potentials and by the conduct of indophenols in aerated cell suspensions would demand of the benzidine reagent an improbable design to permit appreciable formation of a true meriquinone color. If it be remembered that we are now not discussing extreme conditions to which a cell or a cell constituent can be subjected in driving it to yield a color reaction, it will be conceded that the cases are few, if any, in which a cell suspension, in the absence of added peroxide, can oxidize a member of the benzidine series to its *meriquinone.*

Another bit of evidence can be deduced from the data for the hemoglobin-methemoglobin system presented by Conant and Fieser (1925). If mammalian tissue were capable of maintaining an oxidation-reduction intensity distinctly positive to the region suggested by the foregoing facts there would be found in the blood stream very considerable quantities of methemoglobin under normal conditions.

These considerations and the experimental facts previously mentioned converge to the confirmation of the conclusion stated in the sixth paper of this series—that under the conditions specified, the aerated cell suspension has an oxidation-reduction potential near to or in the zone characteristic of the indophenols.

Furthermore, we shall give in a later paper details of an investigation briefly abstracted (1925) in which it was demonstrated that the halogens, which, in a certain sense, exert oxidizing and substituting actions when added to a bacterial suspension under *any* circumstances, lose the greater part of their *toxicity* when added under conditions which prevent the attainment of potentials positive to the zone now under discussion. Adequate discussion of this must be postponed. We cite it here in a somewhat loosely formulated argument to the effect that the potential region distinctly positive to the indophenols is the region in which the bacteriologist finds his oxidative disinfectants. It is also the region in which the chemist performs his "wet combustions." By the same token, the cell would be in danger of "wet combustion" were the full, theoretical potential of the fifth of an atmosphere of oxygen in the air completely utilized. In the language of teleology, it is a fortunate provision that severe checks have been imposed, or else that advantage has been taken of an inherent relative inactivity of molecular oxygen. The problem of oxygen catalysts in biochemistry is, therefore, not merely that of the mechanism of activation, but that of an activation at a rate or at an intensity level which can keep oxygen from exerting its full potential. Consequently this approach to a definition of the potential zone characteristic of aerated cell suspensions

requires of future speculation on biological oxygen catalysts that the catalysts postulated meet a new and more definite criterion.

General Discussion

An experimental difficulty attending the examination of biological material is the disagreement between electrodes of different metals of different surface structure, or between electrodes of the same metal.

We have postponed to this point a discussion of this very important matter for a reason which will appear presently.

In the first place, the courses of potential-change described in our tables and charts are very much alike when the measurements with different electrodes are compared on a large scale map. Thus, if several electrodes in the same suspension of yeast cells are compared, the slopes of the curves which they individually trace may differ somewhat, but each will exhibit the logarithmic form, and all will reach about the same limiting value. Furthermore, electrodes which give potentials widely apart at the early intervals of time tend to approximate more and more, and, if time be allowed, will often come into excellent agreement.

Consequently, certain important facts revealed by the electrode measurements should not be obscured by the comparatively minor discrepancies. But the discrepancies themselves are very important. They sometimes are of an order of magnitude which would have completely upset the precise equilibrium studies described in earlier papers.

As already mentioned, it was not to be expected that the potentials would be stabilized by strongly active, perfectly reversible systems with large poising action.

Now, in the study of the latter systems we have frequently noted that, when the solution contains appreciable quantities of both oxidant and reductant, electrodes of different or of the same metal agree perfectly, but in extremely dilute solutions or in those mixtures in which the ratio of oxidant to reductant approaches zero or infinity, electrodes, which otherwise agreed perfectly, disagree. Often the lapse of time does not bring them into agreement, and yet they often will run parallel in values as the state of the solution is changed. Purposeful induction of such differences has been put to use by Willard & Fenwick (1922) and Van Name & Fenwick (1925) for detecting end-points in electrometric titrations. The converse, the "controlled electrode," has made possible some suggestive investigations by Dixon and Quastel (1923) and by Goard and Rideal (1924).

Thus, electrodes in cell suspensions remind one of the conduct which would be expected of electrodes in solutions containing systems intermediate between those which poise well and those which have no definite effect whatever. This similarity, which can be made the subject of quantitative study only by refinements of technique and apparatus, supports a suggestion which is supplied by other facts. Definite polarization, or the addition of small but definite quantities of an active oxidant, causes a large displacement in potential in cell suspensions; but a very decided and definite recovery occurs which can contend against many successive small doses of oxidant. In some cases to be reported in a later paper, enormous numbers of small doses of oxidant have been automatically added as the cell suspension successively overcame the effects.

It will have been noted that in none of the cases illustrated in this paper is there evidence of static equilibria. The criterion of stability with respect to time, which is one of the most important criterions of significance in potentials observed with definite reversible systems, is lacking. Therefore, we emphasized at the very beginning the readiness with which the indicators adjust to induced levels of reduction intensities. There have been found on the part of the indicators no specific resistances to reduction with respect to time required, and the responsibility for lag has been thrown upon the cell suspensions.

Briefly summarized, the conduct of cell suspensions is as follows:

Deprived of free access of oxygen, the suspension develops a progressively more negative potential. In this course it traverses in order the zones characteristic of reversible indicators. If one or another of these indicators is present, the potentials pass smoothly into equilibrium potentials of the dye system, the dye is progressively reduced, and the potentials then pass smoothly out of the zone characteristic of the indicator and take up their normal course. Successive small doses of oxidant produce temporary checks or reversals upon the course of potential-change followed by a very decided recovery of the negative drift.

In short, the suspensions act as if there were present very small quantities of active poising material fed from a large reserve which is slowly but decidedly mobilized.

Thus a reasonable interpretation of the potentiometric observations we have reported with cell catalysts would seem to be that the catalyst affects the mobilization. The full import of such an effect can not be appreciated until the electrochemical formulaton of oxidation-reduction can be extended to the so-called irreversible systems which comprise the greater part of organic chemistry. At the same time, evidence is accumulating that the electrochemical requirements are satisfied in some semireversible systems, so that we feel that

we have some justification for the suggestion that in the electromotive activations we are discussing we are dealing with intramolecular adjustments of electronic structures into forms to which the electrochemical considerations may be applied. The definition of this activation in structural terms must, however, await the systematization of the structural peculiarities common to electromotively active species. There is hope that the study of the semireversible systems above referred to will go some way toward defining these.

It would seem that there exist catalysts whose function is an activation of a sort that would result in making a substance electromotively active. Indeed, this is partially implied in certain theories of the function of polar catalysts. Perhaps our experiments indicate that it would be worth while to search for evidence of this sort of activation.

However this may be, it will be agreed that we have demonstrated an effect of a tissue upon an added substance where previously the addition of a third system, that of an indicator, alone made possible the observation of the effect, and that this demonstration has been by methods which can reasonably be interpreted as indicative of electron transfers.

There occur various hints that the observed potentials *tend* to record the conduct of truly electromotively active systems subjected to the shifting conditions of the obviously abnormal environment in which the cells are placed. Perhaps the best hint is the parallelism between events as recorded by the electrode and events as revealed by definite, reversible, electromotively active systems, that is, the indicators. When the implications of our data on reversible indicators are appreciated, it becomes almost impossible to conceive that the dyes are reduced by the complicated processes implied in current speculations.

In Figure 3 there is a placement of curves with respect to pH which is somewhat unfortunate in that the apparent order may mislead the casual reader. Without an exhaustive, favorable analysis of the placements with respect to the *abscissa* there would be little justification in using this apparent order wherein the position of each curve with respect to the potentials is in the order of the pH value at which the experiment was made. However, unique points may have some significance. Let us assume that the slope of the first part of the curve is indicative of the velocity with which the system plunges into a more strongly poised region of potential, and that the second section of the curve is indicative of such a region being reached. Obviously, with no knowledge of the effect of pH upon the metabolism within this latter region and with no knowledge which permits the analysis of the kinetic relations in a search for the equilibrium data that may lie concealed within

them, the best that we can do at the moment is to select arbitrarily some unique point of the curve such as the first sign of deviation. That this will bear analysis we are not sure; but from it turns up a hint. In Figure 3 the first point of deviation as judged from the first careful sketch on cross section paper is indicated in each instance by an arrow. The potentials at these points, when plotted against the pH values used, fall as shown by circles in Figure 10. No value for pH 9.25 is given because the experiment in

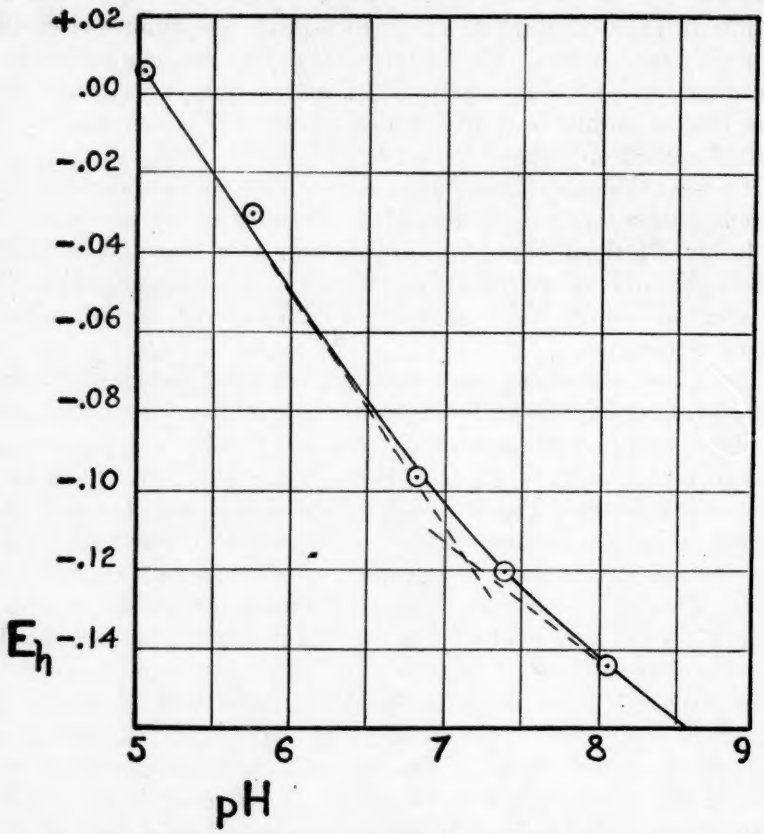

FIG. 10.—Relation between the potentials at points indicated by arrows in Figure 3 and the pH values of the solutions

this borate buffer was obviously abnormal in relation to the other experiments. The curve of this figure is that of a reversible system at any fixed degree of reduction in its relation to pH when a dissociation of one hydrion is centered at pH 7.1 (compare previous articles of this series). The coincidence is, we repeat, a mere hint, but a suggestive one withal.

With bacterial cultures a number of other suggestive facts have been found.

Upon platinized electrodes the potential of a hydrogen electrode is induced in sugar media by cultures such as *Bact. coli* which may, under proper conditions, liberate hydrogen. Perhaps this might be expected as a natural and insignificant consequence of the juxtaposition of hydrogen and platinum black. But, in the present state of our knowledge, it is equally legitimate to suggest that the appearance of hydrogen harmonizes with the attainment of that reduction intensity at which hydrogen should be liberated. Indeed, it seems to require this more comprehensive point of view to maintain perspective when, on the one hand, certain cultures induce a definite overvoltage with the hydrogen which is liberated at considerably less than one atmosphere pressure and when, on the other hand, certain other cultures induce various potentials more positive than that of the hydrogen electrode but no less distinctive.

The first of the facts mentioned above has been confirmed several times and is illustrated in Figure 9. It will require very careful consideration before final judgment is rendered; but if taken at face value it suggests that some system other than $H_2:H^+$ is the determinant, and that the cell which establishes this other system should liberate hydrogen if it possesses a catalyst capable of hastening the attainment of an equilibrium state within the time of an ordinary experiment.

The second of the facts mentioned above completes the spectrum, as it were, of final potentials observed in cell suspensions. These potentials extend without distinctive gaps from those of the aerated suspensions of aerobic cells through the entire range up to and definitely beyond the potentials of a hydrogen electrode.

Such facts suggest that there must be some correlation between the characteristic potentials and the metabolism characteristic of species.

This suggestion recalls the concept of Kluyver and Donker (1925) as presented, for instance, in one of a series of papers. These authors have attempted to establish a fundamental order in bacterial metabolism by postulating specific hydrogen "affinities" for the protoplasm of different species. This extremely hypothetical postulate is developed in terms of Wieland's hypothesis and has led to several conclusions which can be made the subject of severe criticism. However, we believe that the idea can be divested of several implications which have been introduced and can be expressed in terms more nearly amenable to experimental test. Indeed it is a natural consequence of our experimental work that we should look for a correlation between the appearance of certain products and the attainment of certain levels of reduction intensity, and we have long since projected experiments in this direction. Under condi-

tions which we are not yet prepared to say are significant, cultures of different species attain distinctly different final levels of reduction potential; but we would hardly speak of this in terms of specific "affinities" for hydrogen on the part of the protoplasm.

Indeed, it is not at all clear what relation the potentials of a cell suspension bear to conditions within the living intact cell. This is, after all, one of the fundamental problems. Consequently it is all the more important to consider even the most elementary aspects of the potentiometric data in order that ultimately there may be developed clear concepts of what has to be dealt with when conditions within and without the cell are jointly formulated.

The span of potential covered by cell-suspensions under anaerobic conditions is so large that within this span several *homogeneous* reversible oxidation-reduction systems of ordinary type could be placed without serious overlapping. This is illustrated by Figure 2, with its depiction of the spans of several indicators. If it be objected that a false impression of the extent of this span has been created by the precarious nature of the potentials observed or perhaps by the biological reduction dragging a lagging electrode out of a relatively positive region induced by initial oxygen content or perhaps by some other more or less accidental phenomenon, the answer is simple. The suspensions have done more work in overcoming the oxidizing capacity of added indicators than has been imposed by the very slight polarizations of electrodes incident to potentiometric balancing, and this *work* against a capacity has been done over the same span of *intensity* that is measured potentiometrically.

It would be strange were a normal cell to maintain what some writers are pleased to call a dynamic equilibrium between the intake and outgo of oxidants and reductants and yet to spread a well-knit-together chemistry over so wide a zone of potential. An obvious answer is that many cell suspensions which exhibit this span are not representative of the normal environment of the cell, or in case they are, the chemistry is not uniform. The latter we know to be true for certain bacterial cultures. Another answer, and one more important to the progress of the subject, is that the proposal implies at least two assumptions the justification of which has not yet been adequately examined.

One is the implication that the so-called irreversible systems, which there is good reason to believe are the chief systems with which the cell has to deal, can be compared with the well-defined reversible systems of simple type. There is no adequate material on this subject as yet. The other assumption is that heterogeneous systems, such as are undoubtedly present, can be considered in rough

outline by means of the ordinary equations. The most simple aspects of this subject have still to be developed.

Thus our preliminary survey may serve to show that there is very little use in attempting more than the roughest of formulations of the biological problem before it is possible to formulate homogeneous and heterogeneous, reversible and irreversible systems better than can now be done. Therefore the chief problem becomes one of pure chemistry, and until it is solved the theorist who speculates on biochemical oxidation-reduction had best make "discretion the better part of valor."

Tentatively, for purposes of experimental approach only, we suggest that it will be helpful to make a sharp distinction between the events of aerobiosis and those of anaerobiosis. It is conceivable that the intact, aerobic cell under normal conditions maintains a dynamic equilibrium between its reductive tendencies and oxygenations which permits a more or less permanent stabilization of its oxidation-reduction chemistry. If so, there may be found for this situation a more or less tenuous or even a decided index in a definite level of potential such as Needham and Needham (1925) believe they have glimpsed in micro-injected cells. If so, an indiscriminant subjection of the cell to an environment which fits the indicator chosen, e. g., methylene blue, may not help much in the solution of aerobic problems.

Cell *suspensions* under anaerobic conditions not only run through a remarkably wide zone of potential, but, in the case of certain bacteria, support these potentials with such large quantities of material having an indirect electromotive activity, that it hardly seems possible for the intact cell to escape the reflex effect of the condition for which the cell was initially responsible. In the extremes of such circumstances, decided differences in cell chemistry are to be expected, and a "dynamic equilibrium" of the type sometimes postulated for the normal aerobic cell is not imaginable.

If, then, for purposes of experimental approach, it proves to be profitable to make a sharp distinction between the events of true aerobiosis and the events of true anaerobiosis, it will probably be found that certain experimental facts assembled by Wieland (1922) in support of his theory and other experimental facts which led Warburg (1923) to make an absolute denial of the validity of Wieland's argument belong to sufficiently different categories to remove one basis of contention.

Therefore, at the risk of needless repetition, we emphasize the fact that the user of methylene blue has unconsciously and automatically selected a narrow zone of the entire course of reducing intensities taken by various cell suspensions. We now see why this has been.

The methylene blue system occupies a peculiar position with respect to the potentials developed in cell suspensions. Its decoloration indicates a level of reducing intensity not so easy of attainment as to prevent the differentiation of certain phenomena and yet not so extreme as to require inconvenient or impossible periods of time. Indeed were the potential of this dye system displaced much in either direction on the potential scale, it probably would not have been possible to demonstrate several of the phenomena which have been observed with washed tissues, nor would the dye have served in the demonstration of certain peculiar relations of glutathione to tissues.

Now, these advantages of methylene blue should not obscure the suggestive evidence that cell suspensions submitted to methylene blue come rapidly to a position where they are poised distinctly outside the range of aerated suspensions. Therefore we again repeat the query: What relation to true aerobiosis have the events discovered by the use of methylene blue reduction?

The relation often assumed is found in the implications of certain current hypotheses. It is widely held that such enzymes as the Schardinger enzyme of milk, the reductases of yeast, of bacteria and of washed muscle, or the enzymes of whatever name that are supposed to be concerned in reductive processes, act as hydrogen activators or "hydrogen transportases." Wieland postulated such an activation of the hydrogen of a substance presented to the catalyst that it is accepted by the catalyst and by it transferred to the acceptor. Methylene blue or oxygen can be such an "acceptor." In short, so far as the elements of the hypothesis are concerned there is no very clear distinction such as we would draw between oxygen and methylene blue. (Compare Ahlgren.)

In regard to the part played by hydrogen we believe we made it clear in an earlier paper of this series that if the theory is presented only as a schematic coordination of the diverse reactions embraced by catalytic reduction, no qualification need be made; for the hypothesis offers a useful condensation for descriptive purposes. If, on the other hand, there is intended a description of the actual mechanism of the processes, certain relations deserve consideration.

In the first place, as was indicated in the former paper, if the participation of hydrogen per se has any reality, then we must expect that the hypothetical hydrogen pressures corresponding to systems which we have demonstrated are in temporary states of equilibrium (e. g., methylene blue-methylene white) are real, and that they are such that the catalyst can effect a concentration of hydrogen at its surface (i. e., can appreciably cover its surface) sufficient to permit a measurable rate of reaction between hydrogenated catalyst and acceptor. Langmuir has shown that some gases are able to form

monomolecular layers on heated tungsten and platinum filaments at pressures as low as 10^{-6} atmospheres, but there seems to be no data which would permit us to infer a comparable state of affairs when the calculated partial pressure of hydrogen is of the order of 10^{-15} atmosphere. Yet this is the calculated value for half-reduced methylene blue at pH 7. Calculation values indicating no reality whatever are found for the half-reduced indophenols which lie in a region of potential where aerated cell suspensions seem to lie.

Again, it has repeatedly been pointed out in this series of papers that in a reduction wherein the isolated reactants show a difference of two hydrogen atoms these hydrogens may belong no more to the reductant than to any other component of the solution. Thus, at pH 7, although two *equivalents* of hydrogen are concerned in the oxidation-reduction of methylene blue, only one hydrogen becomes fixed in the reductant, the balancing of the equation being effected by means of the ever-present hydrion. If, as the electrode equation implies, the oxidation-reduction process may be expressed in the following form:

$$\overset{+}{\mathrm{Ox}} + \mathrm{H}^+ + 2\mathrm{e} \rightleftharpoons \mathrm{H\,Red}$$

methylene blue becomes no more a hydrogen acceptor than it is an electron acceptor. If the same considerations apply to the donator of the experiments, it would appear that what is effected essentially is an electron transfer to which the hydrogens are secondarily accommodated by exchange of hydrions.

We see no way to reconcile with data now available several of the expressed and implied consequences of Wieland's theory; but, as frequently happens in scientific progress, there is discoverable in his theory a point of view which becomes valuable if carefully interpreted. Perhaps Wieland's activation of hydrogen can be translated in structural terms as such an induced rearrangement of the electronic structure of the molecule that electron exchanges with other substances can take place. It would follow that the *isolated* compounds resulting from such an interchange should, in many instances, differ in hydrogen content because of the rule of electrical neutrality. Experimentally, the actual transformations are measurable in a limited number of cases by methods which may reasonably be regarded as implying electron interchanges. Experimentally, also, the resulting hydrogen differences are in certain instances measurable in terms of the values of hydrion dissociation constants. But, as shown in the first paper of this series, conclusions in regard to mechanism are in large measure intuitively deduced and the only perfectly safe deductions are those which rest in the final thermodynamic equations.

In these the work term as a whole has been made the subject of considerable study in the biological field; but we recall no considerable body of data in which the work term has been factored into its components, intensity and capacity. We look in vain for potentials (in the widest sense of the word) which are measures of the intensity levels determining the direction of reactions between reversible systems. Lacking these, we expect still less in the realm of irreversible reactions. Yet speculators feel free to deal with "affinities" without recognizing the only true measures which theoretical chemistry has provided.

Are, then, the electrode potentials we have described in any way connected with the chemical potentials we need to know?

The very considerable span of potential observed exceeds those potentials which can be attributed to certain other causes, and therefore has an interest as a possibly indirect measure of a source of so-called bioelectric potential differences developed in the organized heterogeneous systems—cells and cell colonies; but its more fundamental importance lies in its possible relation to the intensity levels of the work function which determine the direction of oxidative and reductive reactions.

Definite conclusions in regard to this can be drawn only when it becomes possible adequately to formulate the electromotive behavior of reversible, irreversible, homogeneous, and heterogeneous systems. The physiologically important systems the biochemist must reveal in better detail and the physical chemist must define in the perfection of their isolated detail. Then we can return to a more intelligent study of some of the problems which this investigation has hardly done more than to reveal.

Summary

It is shown that measurements of the potential differences at "unattackable" electrodes immersed in suspensions of cells can be used to follow the progress of reducing intensities in the presence and absence of indicators, "metabolites," glutathione, etc. The detail revealed is far greater than that of uncontrolled indicator measurements.

There are revealed relatively wide spans of potential ranging from the hydrogen overvoltage of *Bact. coli* cultures to the zone +0.1 to +0.2 volts at pH 7.0 found in aerated suspensions. The order of magnitude of the oxidation-reduction intensity in the latter case has been confirmed in several ways and is believed to establish a new criterion to be met in the postulation of biological oxygen catalysts.

Various aspects of the experimental data seem to indicate that cell suspensions are poorly poised with respect to the electromotively

active material present at any moment but that active material is slowly mobilized by cell catalysts from some large reserve.

There are discussed several of the implications of current theories regarding biological oxidation and reduction. This discussion and the experimental data lead to the conclusion that the immediate problem is a clearer definition in *experimental* terms of the isolated chemical systems found in the cell. One experimental method of approach has been provided.

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